PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :		(11) International Publication Number:
C07K 14/705, A61K 38/17, 39/00	A1	WO 97/15597
		(43) International Publication Date: 1 May 1997 (01.05.97)

(21) International Application Number: PCT/NL96/00414

(22) International Filing Date: 24 October 1996 (24.10.96)

(30) Priority Data:
95202893.4 25 October 1995 (25.10.95) EP
(34) Countries for which the regional or international application was filed: NL et al.

(71) Applicant *(for all designated States except US)*: CENTOCOR B.V. [NL/NL]; Einsteinweg 101, NL-2333 CB Leiden (NL).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: WARNAAR, Sven, Olle [NL/NL]; van Beuningenlaan 20, NL-2334 CC Leiden (NL). MELIEF, Cornelis, Joseph, Maria [NL/NL]; Wilhelmina Park 33, NL-2012 KV Haarlem (NL). RAS, Elisabeth [NL/NL]; van der Werfstraat 33, NL-2312 VT Leiden (NL). LITVINOV, Sergey, Victor [NL/NL]; Bonhoeffersingel 26, NL-1069 NB Amsterdam (NL).

(74) Agent: SMULDERS, Th., A., H., J.; Vereenigde Octrooibureaux, Nieuwe Parklaan 97, NL-2587 BN The Hague (NL).

(81) Designated States: CA, JP, US, European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: HUMAN EPITHELIAL ANTIGEN Ep-CAM DERIVED PEPTIDES AND THEIR USE

(57) Abstract

Peptides comprising an amino acid sequence which has the ability to bind to human Major Histocompatibility Complex (MHC) Class I molecules, in particular the MHC Class I allele HLA-A*0201, and especialy those peptides that are capable of inducing a primary cytotoxic T lymphocyte (CTL) response. The amino acid sequence is derived from the protein Ep-CAM. The peptides are useful for treatment or prophylaxis of Ep-CAM positive cells, in particular Ep-CAM associated cancers, for in vivo or in vitro induction of CTLs that are effective against Ep-CAM positive tumor cells, and for diagnostic purposes.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AM	Armenia	**GB**	United Kingdom	**MW**	Malawi
AT	Austria	**GE**	Georgia	**MX**	Mexico
AU	Australia	**GN**	Guinea	**NE**	Niger
BB	Barbados	**GR**	Greece	**NL**	Netherlands
BE	Belgium	**HU**	Hungary	**NO**	Norway
BF	Burkina Faso	**IE**	Ireland	**NZ**	New Zealand
BG	Bulgaria	**IT**	Italy	**PL**	Poland
BJ	Benin	**JP**	Japan	**PT**	Portugal
BR	Brazil	**KE**	Kenya	**RO**	Romania
BY	Belarus	**KG**	Kyrgystan	**RU**	Russian Federation
CA	Canada	**KP**	Democratic People's Republic of Korea	**SD**	Sudan
CF	Central African Republic			**SE**	Sweden
CG	Congo	**KR**	Republic of Korea	**SG**	Singapore
CH	Switzerland	**KZ**	Kazakhstan	**SI**	Slovenia
CI	Côte d'Ivoire	**LI**	Liechtenstein	**SK**	Slovakia
CM	Cameroon	**LK**	Sri Lanka	**SN**	Senegal
CN	China	**LR**	Liberia	**SZ**	Swaziland
CS	Czechoslovakia	**LT**	Lithuania	**TD**	Chad
CZ	Czech Republic	**LU**	Luxembourg	**TG**	Togo
DE	Germany	**LV**	Latvia	**TJ**	Tajikistan
DK	Denmark	**MC**	Monaco	**TT**	Trinidad and Tobago
EE	Estonia	**MD**	Republic of Moldova	**UA**	Ukraine
ES	Spain	**MG**	Madagascar	**UG**	Uganda
FI	Finland	**ML**	Mali	**US**	United States of America
FR	France	**MN**	Mongolia	**UZ**	Uzbekistan
GA	Gabon	**MR**	Mauritania	**VN**	Viet Nam

Title: Human epithelial antigen Ep-CAM derived peptides and their use

FIELD OF THE INVENTION

The subject of the invention lies in the field of malignant diseases and more specifically in the field of carcinomas, i.e. tumors that originate from epithelial cells.

The present invention provides new peptides, derived from a widely occurring normal epithelial and carcinoma associated protein, and the use of such peptides in the treatment of cancer patients. Pharmaceutical compositions comprising the peptides described above are also disclosed.

The peptides of the invention are derived from the protein sequence of the human epithelial cell adhesion molecule Ep-CAM and can be used in the prophylaxis or treatment of Ep-CAM positive tumors and other diseases.

The peptides of Ep-CAM comprise amino acid sequences for use in human T cell response-inducing compositions.

BACKGROUND OF THE INVENTION

The human epithelial antigen Ep-CAM (1), also known as EGP40, 17-1A antigen, ESA, etc., is a 40 kD transmembrane glycoprotein that is expressed on the majority of simple cuboidal or columnar, pseudo stratified columnar as well as transitional epithelia (2-5) but not on most squamous epithelia.

Expression of the Ep-CAM protein is also found on tumors arising from the epithelia expressing Ep-CAM, as well as on most squamous carcinomas where Ep-CAM can be regarded as a "de novo" protein related to the proliferative or malignant state of the cells.

The Ep-CAM protein is a cell adhesion molecule normally expressed on the lateral sides of adjacent epithelial cells (1). The protein is quite conserved in evolution with 86% amino acid homology between human and murine Ep-CAM (6). The gene encoding Ep-CAM has been cloned as the GA733-2 gene (7-9).

The expression of the Ep-CAM protein on a wide range of human carcinoma cells have made the protein an attractive target for immunotherapy with monoclonal antibodies (Mabs) (10-14).

Monoclonal antibodies against Ep-CAM have been used either as "naked" IgGs, as radio conjugates (10, 11), or as immunotoxins (12), in which antibody was chemically coupled to toxins or cytotoxic drugs. Radio-immunotherapy has also been investigated (14). From these studies it became apparent that advanced carcinoma cannot be adequately treated with anti-Ep-CAM Mab therapies. However, in a minimal residual disease setting, where tumor loads are low, treatment of patients, at risk for recurrence, with the anti Ep-CAM monoclonal antibody 17-1A was shown to be effective in reducing tumor recurrence, with an improvement in 5 year survival from 50 to 65% in Dukes C colon carcinoma patients, whose primary tumors had been surgically removed (15). The mechanism of action, although unproven, most likely was antibody-dependent cellular cytotoxicity (ADCC), but other mechanisms like complement-mediated cytotoxicity or indirect effects through perturbation of the anti-idiotypic network leading to induction of Ab3, may also have contributed (14).

From these studies it can be concluded that an antibody reacting with a normal tissue component can be used safely and effectively to combat tumor cells that carry the antigen recognized by the antibody.

In animal studies it has been demonstrated that the most effective antitumor immune therapies involve tumor-specific cytotoxic T cells. Induction of specific T cells in tumor-bearing animals, or passive infusion of such cells, was shown to be effective in eradicating established tumors even when these had reached significant proportions (16). In fact, cytotoxic antitumor T cells have been described as being the most effective micro-surgeons.

Therefore, and in view of the current lack of effective immune therapy approaches against most cancers, the generation of cytotoxic T cells capable of attacking Ep-CAM positive tumor cells would seem to be highly desirable. Based on the proven safety of anti-Ep-CAM antibodies it is expected that such anti-

Ep-CAM T cells will have an acceptable safety profile, despite the presence of Ep-CAM on normal epithelial cells.

In view of the dismal prospects of patients suffering from advanced carcinoma, some adverse side effects, due to a reaction with normal tissues, may be acceptable. Most likely they can be managed, if need be, in similar ways as other autoimmune T cell-based diseases.

T cells recognize target cells through a specific receptor-complex called the T cell receptor. The actual ligand recognized by the cytotoxic T cell receptor is a peptide, usually consisting of 8-11 amino acids, in association with a Class I Major Histocompatibility (MHC) molecule (reviewed in 17).

Class I MHC molecules are present on all nucleated cells and can be found in a number of slightly different variants called alleles. Each individual human being has a genetically defined limited set of MHC molecules per chromosome, called the haplotype. Each type of MHC molecule can bind a distinct set of peptides and cannot bind others that may however be bound by other MHC molecules.

Therefore, specific T cells directed against the Ep-CAM molecule that may have developed in a person having a particular haplotype will react with Ep-CAM derived peptides, associated with the particular MHC molecules of that haplotype, that are present on the cell surface of the target cells.

It has been demonstrated that an immune reaction, including the development of antigen-specific cytotoxic and helper T cells, can be effectively triggered by peptides of between 8 and 15 amino acids derived from the antigen. In fact, peptides of an optimal length to fit the binding sides of the MHC molecules were shown to be up to 1000-fold more immunogenic than larger peptides derived from the complete antigen molecules (18). Such peptides can therefore serve as very effective means for vaccination.

In order to define Ep-CAM peptides that can be used to stimulate the development of Ep-CAM specific cytotoxic T cells, it is necessary to establish which peptides can bind to a given MHC class I allele. The frequency of occurrence in the general population of any of the multitude of possible alleles is not

random. A few alleles are widely present and the definition of peptides reacting with the 10 most frequently occurring alleles would allow development of peptide-specific cytotoxic T cells in the vast majority of patients.

In fact, the MHC class I molecule HLA-A*0201 is found in approximately 40% of Caucasians and any Ep-CAM peptides that could bind to this particular allele and lead to T cell activation could be useful as a vaccine against Ep-CAM positive tumor cells in HLA-A*0201 positive patients.

SUMMARY OF THE INVENTION

This invention describes peptides comprising amino acid sequences derived from Ep-CAM that have the ability to bind to the MHC class I allele HLA-A*0201 with high affinities and low dissociation rates (19, 20).

Using the amino acid sequence of the Ep-CAM (= GA 733-2, ESA, etc.) molecule, an overlapping set of 9-mer peptides with an overlap of 8 amino acids was synthesized. In addition motif bearing peptides were selected and synthesized using the binding motif containing glutamin as an anchor at the second position (Drijfhout, personal communication).

The peptides were subjected to two assays studying the binding to the HLA-A*0201 molecule and one test investigating the stability of the complex composed of the peptide and the HLA molecule. These assays were aimed at investigating which peptides could possibly be immunogenic. A predisposition for a peptide being immunogenic is that it can bind in the groove of the MHC molecule. Furthermore, it has been demonstrated that the immunogenicity of a peptide can be predicted by the peptide-induced stability (21).

The first test, that was performed for each peptide, uses the 174CEM.T2 cell line (abbreviated T2) to determine the capacity of the peptides to bind to HLA-A*0201 molecules. The T2 cell line has empty HLA-A*0201 molecules on the cell surface that are therefore unstable. As a consequence a reduced presence of these HLA molecules on the cell surface is observed. Binding of peptide will stabilize the HLA-A*0201 molecules and will lead to an enhanced level of HLA-A*0201 molecules on peptide exposed

cells. This enhancement can be quantified and can be used to detect peptide binding.

A second binding assay was performed with the subset of peptides that bound to the T2 cell line. The HLA class I-peptide complexes on intact human Epstein Barr Virus (EBV)-transformed B cells were stripped and removed by mild acid treatment. The cells were then incubated with a fluorescein (fl)-labeled reference peptide together with different concentrations of the peptide of interest. The effectiveness by which the latter competes for binding to the HLA class I molecules is assayed by measuring the amount of HLA-bound fluorescent peptide.

A third test consisted of measurement of the stability of MHC-peptide complexes as a function of time as compared to the stability of complexes induced by a consensus peptide sequence derived from hepatitis B virus, that is known to be immunogenic.

From the combined tests described above 5 peptides were selected that have high binding affinities to and low dissociation rates from the MHC class I allele HLA-A*0201.

An object of the present invention is to provide synthetic peptides which can be used for prevention, prophylaxis, therapy or treatment of carcinoma patients.

DETAILED DESCRIPTION OF THE INVENTION

This invention provides peptides comprising an amino acid sequence derived from the Ep-CAM protein, wherein said amino acid sequence has the ability to bind to a human MHC Class I molecule, such as particularly HLA-A*0201.

The present invention also provides specific peptides derived from the amino acid sequence of Ep-CAM which, because of their capability to bind to a human MHC Class I molecule, such as particularly the HLA-A*0201 molecule, are candidate peptides to be included in human vaccines that can induce protective or therapeutic T cell responses against Ep-CAM positive tumors.

The peptides of the present invention are useful in pharmaceutical compositions, as screening tools (diagnostics) and in the prevention, prophylaxis, therapy and treatment of carcinoma or other pathological conditions which could benefit

from the peptide-induced CTL reactive to cells exposing the human epithelial antigen Ep-CAM.

Preferably, said amino acid sequences have the ability to bind to human MHC Class I allele HLA-A*0201.

More specifically, this invention provides peptides comprising an amino acid sequence derived from Ep-CAM, wherein said amino acid sequences have the ability to bind to human MHC Class I allele HLA-A*0201 and are selected from the group consisting of:

Q V L A F G L L L	(SEQ ID NO:1)
Y Q L D P K F I T S I	(SEQ ID NO:2)
I L Y E N N V I T	(SEQ ID NO:3)
G L K A G V I A V	(SEQ ID NO:4)
V V A G I V V L V	(SEQ ID NO:5)

and a fragment, homolog, isoform, derivative, genetic variant or conservative variant of any one of these amino acid sequences which has the ability to bind to the human MHC Class I allele HLA-A*0201.

This invention further provides a pharmaceutical composition containing a prophylactically or therapeutically effective amount of a peptide according to the invention, and a pharmaceutically acceptable carrier, diluent, excipient or adjuvant. Preferably, said pharmaceutical composition contains a peptide according to the invention which is able to induce a T cell response effective against Ep-CAM positive tumor cells, in particular a HLA class I-restricted CD8+ cytotoxic T cell response.

In addition, this invention provides a method of prophylactic or therapeutic treatment of Ep-CAM positive carcinoma, comprising administering to said human individual a prophylactically or therapeutically effective amount of a peptide according to the invention which is able to induce a T cell response effective against Ep-CAM positive tumor cells, in particular a HLA class I-restricted CD8+ cytotoxic T cell response.

The invention is directed to peptides comprising an amino acid sequence derived from proteins of Ep-CAM, wherein said amino acid sequence has the ability to bind to human HLA molecules, such as preferably HLA-A*0201 molecules. In view of our own experience with other antigens, the best candidates for induction of HLA class I restricted CD8+ cytotoxic T cells are the strongest binding peptides.

A most preferred embodiment of the invention concerns peptides comprising an amino acid sequence derived from Ep-CAM, wherein said amino acid sequence has the ability to bind to human MHC Class I allele HLA-A*0201. Specifically, such peptides comprise the following amino acid sequences derived from the noted regions of Ep-CAM (see Table II; the amino acids are identified by the one-letter code of amino acids).

HLA-A*0201 binding peptides were found using a set of 306 peptides spanning the whole Ep-CAM protein consisting of 9 amino acids with an overlap of 8 amino acids. One 11 mer peptide was found after testing 8 longer and shorter peptides that fulfill the new motif requirements, with Q as second amino acid (Drijfhout, personal communication).

The data suggest that the peptides mentioned above are single polypeptides of identified sequences. However, homologs, isoforms or genetic variants of these peptides may exist in nature. This invention encompasses all such homologs, isoforms or genetic variants of the above peptides provided that they bind to a human HLA molecule, such as preferably the HLA-A*0201 molecule.

Polypeptides that are homologs of the peptides specifically include those having amino acid sequences which are at least 40% conserved in relation to the amino acid sequence set forth in Table II, preferentially at least about 60% conserved, and more preferentially at least about 75% conserved.

It will be understood by one of ordinary skill in the art that other variants of the peptides shown above are included within the scope of the present invention. This particularly includes any variants that differ from the above mentioned and synthesized peptides only by conservative amino acid

substitution. Many such conservative amino acid substitutions are set forth by Taylor (22).

Herein the peptides shown above or fragments thereof include any variation in the amino acid sequence, whether by conservative amino acid substitution, deletion, or other processes, provided that the polypeptides bind to a human HLA molecule, particularly the HLA-A*0201 molecule. The fragments of the peptides may be small peptides with sequences of as little as five or more amino acids, said sequences being derived from those disclosed in Table II, when said fragments bind to HLA-A*0201 molecule.

Polypeptides larger than the peptides shown are especially included within the scope of the present invention when said polypeptides induce an Ep-CAM specific CTL response, in particular in HLA-A*0201 positive individuals, and include a (partial) amino acid sequence as set forth in Table II, or conservative substitutions thereof. Such polypeptides may have a length up to about 30 amino acids, preferably up to about 27 amino acids. Most preferably they have a length of 9 to 11 or 9 to 10 amino acids.

This invention includes the use of polypeptides generated by every means, whether genetic engineering, peptide synthesis with solid phase techniques or others. The foregoing peptides may have various chemical modifications made at the terminal ends and still be within the scope of the present invention. Also other chemical modifications are possible, particularly cyclic, dimeric, polymeric, or tandem repeat configurations. The term "derivatives" intends to cover all such modified peptides.

The polypeptides of the present invention find utility for the treatment of carcinoma or the prevention of cancer or cancer recurrence.

For all applications the peptides are administered in an immunogenic form. Since the peptides are relatively short, this may necessitate conjugation with an immunogenicity conferring carrier material such as lipids or others, or the use of adjuvants.

The magnitude of a prophylactic or a therapeutic dose of polypeptides of this invention will, of course, vary with the

group of patients (age, sex, weight, etc), the nature of the severity of the condition to be treated, the particular polypeptide of this invention and its route of administration. Any suitable route of administration may be employed to achieve an effective dosage of a polypeptide identified by this invention, as well as any dosage form well known in the art of pharmacy. In addition the polypeptides may also be administered by controlled-release means and/or delivery devices. They may also be administered in combination with other active substances, such as, in particular, T cell-activating agents like interleukin-2 etc.

The peptides of this invention may also be useful for other purposes, such as diagnostic uses. For example, they may be used to check whether a vaccination with a peptide according to the invention has been successful. This may be performed by in vitro testing whether said peptides are able to activate T cells of the vaccinated person. In addition said peptides may be used in in vitro methods to elicit or to expand HLA*0201 restricted T cells with an HLA-restricted specificity against the Ep-CAM molecule. The T cells generated in vitro can be used for therapy of patients as will be obvious to a person skilled in the art.

Brief description of the technical data/methods used

Table I gives the result of the binding analysis of 8 Ep-CAM related, 9 aminoacid long peptides and one 11 mer, to HLA-A*0201 expressed on 174CEM.T2 cells. The results of the additional competition and dissociation tests performed are also shown.

The peptides presented in Table I include all 9 mer peptides from the overlapping set that bind to the HLA-A*0201 haplotype. Two non-binding peptides are also listed as negative controls. In addition the one peptide (out of 8 tested) with Q as second amino acid that bound to HLA-A*0201 is included. This is the peptide with residues 174-184.

The binding of each peptide to the HLA-A*0201 molecule was determined for a range of concentrations. The Fluorescence Index (FI) was calculated for each value. The FI is determined as follows: (mean fluorescence T2 cells + peptide/mean fluorescence

T2 cells - peptide) - 1. Binding of a peptide to the HLA-A*0201 molecule was regarded positive when the FI was equal to or higher than 0.5 at a concentration of 100 ug peptide per ml medium. Two peptides (010-018 and 274-282) did not bind and are included in Table I as representative non-binding peptides.

The values in the column "IC50" are given as inhibitory concentration 50 (IC50), and represent the amount of peptide (in uM) needed to inhibit 50% of the signal of the fluorescent consensus peptide, derived from the hepatitis B core protein, amino acids 18-27, FLPSDC(fl)FPSV in single letter code. The last column shows values concerning the stability of the peptide-MHC complex in hours compared to the stability of the reference peptide (hepatitis B core protein, amino acids 18-27, FLPSDYFPSV in single letter code).

The binding and stable peptides are numbered 1 to 5; this numbering corresponds to the numbering in Table II.

The following examples illustrate the present invention without limiting the same thereto.

EXAMPLE 1

Peptide synthesis: Materials

Peptide synthesizer: Abimed AMS 422 (Abimed Analysen-Technik GmbH, Langenfeld, Germany).

Synthesis polymer: Tentagel S AC (0.17-024 meq/g, Rapp Polymere, Tübingen, Germany).

HPLC equipment: The HPLC system used for analysis and purification of peptides consisted of: autosampler 2157, HPLC pump 2248, variable wavelength monitor VWM 2141, column oven 2155, low pressure mixer, all of Pharmacia Nederland B.V., Woerden, The Netherlands, a Star LC-20 dot matrix printer, Star Micronics Co.Ltd, all parts controlled by a Tandon PCAs1/386sx computer, Tandon Computer Benelux B.V., Amsterdam, The Netherlands.

Lyophilizer: Virtis Centry, The Virtis Company, Inc., Gardiner (NY), USA, equipped with an Alcatel 350C vacuumpump, Alcatel CIT, Malakoff, France, connected to a Christ Alpha RVC vacuo-spin, Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany.

Centrifuge: MSE Mistral 6L, Beun de Ronde, Abcoude, The Netherlands.

Mass spectrometer: Bioion plasma desorption mass spectrometer (PDMS), Applied Biosystems, Inc., Foster City (CA), USA.

Amino acid Analysis: HP Aminoquant, Hewlett Packard, Amstelveen, The Netherlands.

Chemicals: All chemicals were used without further purification unless stated otherwise.

Fmoc (9-fluorenylmethyloxycarbonyl) amino acids were of the L-configuration, bearing the following side chain protecting groups: t-Bu (tert-butyl) for Asp, Glu, Tyr, Ser and Thr; Trt (trityl) for His, Asn and Gln; Pmc (2,2,5,7,8-pentamethyl-chroman-6-sulfonyl) for Arg; Boc (tert-butyloxycarbonyl) for Lys; all Novasyn and purchased from Pharmacia Nederland B.V., Woerden, The Netherlands.

Piperidine was purchased from Aldrich Chemie Benelux N.V., Brussels, Belgium.

BOP (benzotriazole-1-yl-oxy-tris-(dimethylamino) phosphonium hexafluorophosphate) was obtained from Richelieu Biotechnologies, St-Hyacinthe, Canada.

N-methylmorpholin (NMM, Janssen Chimica, Tilburg, The Netherlands) was distilled from NaOH at atmospheric pressure before use.

N-methylpyrrolidone (NMP, Aldrich Chemie) was vacuum distilled under a nitrogen atmosphere (b.p. 78-80°C, 18 mm Hg) before use.

Acetonitrile (HPLC grade) was purchased from Rathburn Chemicals Ltd., Walkerburn, Scotland.

Ether (Baker Analyzed Grade), pentane (Baker Grade) and acetic acid (Baker Analyzed Grade) were purchased from J.T. Baker B.V., Deventer, The Netherlands.

Ethanethiol was obtained from Fluka Chemie, Brussels, Belgium.

Dichloromethane and N,N-dimethylacetamide (DMA) were purchased from Janssen Chimica, Tilburg, The Netherlands.

Trifluoroacetic acid (TFA, z.S. grade) was obtained from Merck-Schuchardt, Hohenbrunn, Germany.

Disposables: Polypropylene reaction vessels containing a PTFE filter were purchased from Abimed Analysen-Technik GmbH, Langenfeld, Germany. All other disposables used were made of polypropylene and obtained from Sarstedt B.V., Etten-Leur, The Netherlands.

Experimental conditions: All experiments were performed at room temperature unless stated otherwise. All Fmoc protected amino acids, synthesis polymers, peptides and TFA were stored at -20°C.

Peptide synthesis: Methods

Peptides were synthesized by solid phase strategies on an automated multiple peptide synthesizer (Abimed AMS 422; 23, 24).

The peptides were made in various runs, in each of which 48 different peptides were synthesized simultaneously.

Tentagel S AC (25, 26), a graft polymer of polyethylene-glycol spacer arms on a polystyrene matrix, was used as a resin (40-60 mg per peptide, 10 umol Fmoc amino acid loading).

Repetitive couplings were performed by adding a mixture of 90 ul 0.67 M BOP (27, 28) in NMP, 20 ul NMM in NMP 2/1 (v/v) and 100 ul of an 0.60 M solution of the appropriate Fmoc amino acid (29) in NMP (6-fold excess) to each reaction vessel. At 70% of the reaction time approximately 50 ul dichloromethane was added to each reaction vessel.

Fmoc-deprotection was performed by adding 3 times 0.8 ml of piperidine/DMA 1/4 (v/v) to each reaction vessel.

Coupling and deprotection times were increased as the synthesis proceeded, starting with 30 min and 3 times 3 min respectively.

Washings after couplings and Fmoc-deprotections were done with 6 times 1.2 ml DMA. After the required sequence had been reached and the last Fmoc-protection was removed the peptidyl-resin was washed extensively with DMA, dichloromethane, di-chloromethane/ether 1/1 (v/v) and ether respectively, and dried.

Peptide cleavage and isolation

Cleavage of the peptides from the resin and removal of the side chain protecting groups was performed by adding 6x 200 ul

TFA/water 19/1 (v/v) at 5 min intervals to each reaction vessel, thus yielding free carboxylic peptides. For Trp-containing peptides TFA/water/ethanethiol 18/1/1 (v/v/v) was used.

Two hours after the first TFA addition the peptides were precipitated from the combined filtrates by addition of 10 ml ether/pentane 1/1 (v/v) and cooling to -20°C. The peptides were isolated by centrifugation (-20°C, 2500g, 10 min).

After treatment of the pellet with ether/pentane 1/1 (v/v) and isolation by the same centrifugation procedure, the peptides were dried at 45°C for 15 min.

Each of the peptides was dissolved in 2 ml water (or 2 ml 10 vol.% acetic acid), the solution frozen in liquid nitrogen for 3 min, and lyophilized while being centrifuged (1300 rpm, 8-16 h).

Analysis and purification

The purity of the peptides was determined by reversed phase HPLC; an aliquot of about 50 nmol was dissolved in 100 ul 30 vol.% acetic acid. Of this solution 30 ul was applied to an RP-HPLC system equipped with a ternary solvent system; A: water, B: acetonitrile, C: 2 vol.% TFA in water.

Gradient elution (1.0 ml/min) was performed from 90% A, 5% B, 5% C to 20% A, 75% B, 5% C in 30 min. Detection was performed at 214 nm.

Samples taken at random were analyzed by mass spectrometry on a PDMS. The 5 binding peptides were all analyzed by mass spectrometry on a PDMS and by quantitative amino acid analysis after hydrolysis on a HP Aminoquant. Of all analysed samples the difference between calculated and measured masses was within the experimental error (0.1%) as specified by the producer of the equipment used. All amino acid compositions were as expected.

EXAMPLE 2

Binding assay using the 174CEM.T2 cell line

Peptides

Of all 314 Ep-CAM peptides that had been freeze dried, 1 mg was weighed and dissolved in 10 ul of DMSO. Of all dissolved peptides a dilution of 4 mg/ml in 0.9% NaCl was made and the pH was neutralized to pH 7 with 5% acetic acid diluted in distilled water (CH_3COOH, Merck Darmstadt, Germany: 56-1000) or 1N NaOH diluted in distilled water (Merck Darmstadt, Germany: 6498).

Cells

174CEM.T2 cells were cultures in ISCOVE's modified Dulbecco's medium (Biochrom KG Seromed, Berlin, Germany: F0465) supplemented with 50 IU/ml penicillin and 50 ug/ml streptomycin (Gibco, Paisley, Scotland: 15070-022), 2mM glutamine (l-glutamin 200mM (100x), Gibco, Paisley, Scotland: 25030-024) and 10% fetal calf serum (FCS, Hyclone Laboratories Inc. Logan, Utah, USA: A-1115-L). Cells were cultured at a density of 2.5 x 10^5/ml during 3 days at 37°C, 5% CO_2 in humified air.

Peptide binding

The 174CEM.T2 cell line expresses "empty" and unstable HLA-A*0201 molecules that can be stabilized when a peptide is binding to the peptide presenting groove of these molecules. A stabilized HLA-A*0201 molecule that will not easily degrade is the result of binding of an analyzed peptide. This leads to an increase in cell surface expression of the HLA-A*0201 molecule, which can be detected using a fluorescent anti-HLA-A2 antibody.

174CEM.T2 cells were washed twice in culture medium without FCS and put in serum-free culture medium to a density of 2 x 10^6 cells/ml. Of this suspension 40 ul was put into a V-bottomed 96 well plate (Greiner GmbH, Frickenhausen, Germany: 651101) together with 10 ul of twofold serial dilutions in 0.9% NaCl of the individual peptide dilutions (ranging from 500 ug/ml). The end concentrations range from 200 ug/ml to 3.1 ug/ml peptide with 8 x 10^4 174CEM.T2 cells. This solution was gently agitated for 3 minutes and was then incubated at 37°C, 5% CO_2 in humified air for 16 hours. Then cells were washed once with 100 ul 0.9%

NaCl, 0.5% bovine serum albumin (Sigma St. Louis, USA: A-7409), 0.02% NaN_3 (Merck Darmstadt, Germany: 822335). After a centrifuge round of 1200 rpm the pellet was resuspended in 50 ul of saturating amounts of HLA-A2 specific mouse monoclonal antibody BB7.2 for 30 minutes at 4°C. Then cells were washed twice and incubated for 30 minutes with $F(ab)_2$ fragments of goat anti-mouse IgG that had been conjugated with fluoresceine isothiocyanate (Boehringer Mannheim, Mannheim, Germany: 605240) in a dilution of 1:40 and a total of 25 ul.

After the last incubation, cells were washed twice and fluorescence was measured at 488 nanometer on a FACScan flowcytomer (Becton Dickinson, Franklin Lakes, NJ, USA). Based on the mean fluorescence values obtained from the flowcytometer the Fluorescence Index (FI) was calculated according to the following formula:

MF= Mean Fluorescence

$$\text{FI= Fluorescence Index=} \frac{\text{(MF) T2 + peptide}}{\text{(MF) T2 - peptide}} - 1$$

The Fluorescence Index of the background fluorescence is 0.

Results

In order to identify Ep-CAM peptides that could bind to HLA-A*0201 molecules expressed by 174CEM.T2 cells two groups of peptides were tested. A set of 306 overlapping peptides, each 9 amino acids long, with an overlap of 8 residues was synthesized and examined. Secondly, a total of eight peptides, 8, 10 and 11 amino acids long, with glutamin at position 2 as an anchor for binding to HLA-A*0201 (Drijfhout, personal communication) were synthesized. Out of the 314 peptides tested for binding to the HLA-A*0201 molecules on the T2 cell line, only 7 met the criterium of expressing a FI equal to or higher than 0.5. These binding peptides are shown in Table 1 together with 2 non-binding peptides that served as negative controls for the assays. Further analysis of the binding sequences was performed using a competition assay and a stability test.

The tests that have been used study either the association of the peptide and HLA, or the dissociation, or both phenomena, respectively. Both association and dissociation of peptide-HLA complexes will occur in the T2 assay that consists of an incubation of 15-18 hours at 37°C. The HLA molecules to which no peptide is bound disintegrate at this temperature. Moreover, dissociation of peptides from the HLA molecules is faster at 37°C than at 4°C. The competition assay is performed overnight at 4°C at which temperature dissociation is low. This test therefore merely studies the association between the peptide and the HLA molecule. The dissociation of peptides is assayed at 37°C and measures the dissociation of the peptide-HLA complexes compared to a reference peptide and t=0. Thus, a peptide with a high FI (as measured in the T2 assay) is likely to have a good association (as measured in the competition assay) and/or good stability (as measured in the dissociation assay). On the other hand, when a peptide shows a low FI this might be explained by either a weak capacity to associate with the HLA molecule to which it can potentially bind, or a low stability, or both.

Only the peptides shown in Table II could bind to T2 cells with a FI equal to or higher than 0.5 (18), competed with the fluorescent-labeled consensus peptide (19) at a concentration lower than 15 uM peptide and were stable for over 6 hours (20). None of the other peptides tested fulfilled these requirements.

These experiments indicate that only a limited proportion of peptides that were synthesized have the ability to bind to the HLA-A*0201 molecule with high affinity (e.g. with a high association rate and a low dissociation rate). Therefore, the peptides depicted in Table II are the most likely Ep-CAM derived sequences to be recognized by human CTL, because CTL recognize peptides only when bound to HLA molecules.

TABLE I

Binding capacity of Ep-CAM derived peptides to the HLA-A*0201 molecule and stability of this complex.

Peptide no.	Residues	FI	IC50	Stability
1	005-013	1.2	3	>6.0
	010-018	0.0	2	2.5
	093-101	0.5	35	1.5
	114-122	0.7	5	4.0
2	174-184	1.5	2	>6.0
3	184-192	1.5	7	>6.0
4	263-271	1.6	5	>6.0
	274-282	0.0	>100	0.0
5	279-287	1.0	7->50	>6.0

TABLE II

Peptides derived from Ep-CAM binding to the HLA-A*0201 molecule.

Peptide	Amino acid sequence	Residues	Seq id no
1	Q V L A F G L L L	005-013	1
2	Y Q L D P K F I T S I	174-184	2
3	I L Y E N N V I T	184-192	3
4	G L K A G V I A V	263-271	4
5	V V A G I V V L V	279-287	5

REFERENCES

1. Litvinov SV, Velders MP, Bakker HAM, Fleuren GJ, Warnaar SO: Ep-CAM: A human epithelial antigen is a homophilic cell-cell adhesion molecule. J Cell Biol 125: 437-446, 1994.

2. Spurr NK, Durbin H, Sheer D, Parkar M, Bobrow L, Bodmer WF: Characterization and chromosomal assignment of a human cell surface antigen defined by the monoclonal antibody AUA1. Int J Cancer 38: 631-636, 1986.

3. Edwards DP, Grzyb KT, Dressler LG, Mansel RE, Zava DT, Sledge Jr GW, McGuire WL: Monoclonal antibody identification and characterization of a Mr 43,000 membrane glycoprotein associated with human breast cancer. Cancer Res 46: 1306-1317, 1986.

4. Momburg F, Moldenhauer G, Hämmerling GJ, Möller P: Immunohistochemical study of the expression of a Mr 34,000 human epithelium-specific surface glycoprotein in normal and malignant tissues. Cancer Res 47: 2883-2891, 1987.

5. Bumol TF, Marder P, de Herdt SJ, Borowitz MJ, Apelgren LD: Characterization of the human tumor and normal tissue reactivity of the KS1/4 monoclonal antibody. Hybridoma 7: 407-415, 1988.

6. Bergsagel PL, Victor-Kobrin C, Timblin CR, Trepel J, Kuehl WM: A murine cDNA encodes a pan-epithelial glycoprotein that is also expressed on plasma cells. J Immunol 148: 590-596, 1992.

7. Strnad J, Hamilton AE, Beavers LS, Gambos GC, Apelgren LD, Taber LD, Sportsman JR, Bumol TF, Sharp JD, Gadski RA: Molecular cloning and characterization of a human adenocarcinoma/epithelial cell surface antigen complementary DNA. Cancer Res 49: 314-317, 1989.

8. Perez MS, Walker LE: Isolation and characterization of a cDNA encoding the KS1/4 epithelial carcinoma marker. J Haematol 142: 3662-3667, 1989.

9. Szala S, Froenlich M, Scollon M, Kasai Y, Steplewski Z, Koprowski H, Linnenbach AJ: Molecular cloning of cDNA for the carcinoma-associated antigen GA733-2. Proc Natl Acad Sci USA 87: 3542-3546, 1990.

10. Ragnhammar P, Fagerberg J, Frödin JE, Hjelm AL, Lindemalm C, Magnusson I, Masucci G, Mellstedt H: Effect of monoclonal antibody 17-1A and GM-CSF in patients with advanced colorectal

carcinoma - long-lasting, complete remissions can be induced. Int J Cancer 53: 751-758, 1993.

11. Weiner LM, Moldofsky PJ, Gatenby RA, O'Dwyer J, O'Brien J, Litwin S, Comis RL: Antibody delivery and effector cell activation in a phase II trial of recombinant γ-interferon and the murine monoclonal antibody CO17-1A in advanced colorectal carcinoma. Cancer Res 48: 2568-2573, 1988.

12. Elias DJ, Kline LE, Robbins BA, Johnson Jr HCL, Pekny K, Benz M, Robb JA, Walker LE, Kosty M, Dillman RO: Monoclonal antibody KS1/4-methotrexate immunoconjugate studies in non-small cell lung carcinoma. Am J Respir Crit Care Med 150: 1114-1122, 1994.

13. Jacobs AJ, Fer M, Fu Min Su, Breitz H, Thompson J, Goodgold H, Cain J, Heaps J, Weiden P: A phase I trial of a rhenium 186-labeled monoclonal antibody administered intra peritoneally in ovarian carcinoma: toxicity and clinical response. Obstetrics/ Gynecol 82: 586-593, 1993.

14. Herlyn D, Harris D, Zaloudik J, Sperlagh M, Maruyama H, Jacob L, Kieny MP, Scheck S, Somasundaram R, Hart E, Ertl H, Mastrangelo M: Immunomodulatory activity of monoclonal anti-idiotypic antibody to anti-colorectal carcinoma antibody CO17-1A in animals and patients. J Imm Ther 15: 303-311, 1994.

15. Riethmüller G, Schneider-Gädicke E, Schlimok G, Schmiegel W, Raab R, Höffken K, Gruber R, Pichlmaier H, Hirche H, Pichlmayr R, Buggisch P, Witte J, and the German Cancer Aid 17-1A Study Group: Randomized trial of monoclonal antibody for adjuvant therapy of resected Dukes' C colorectal carcinoma. The Lancet, Vol. 343, No.8907 Pages 1177-1183, 1994.

16. Kast WM, Offringa R, Peters PJ, Voordouw AC, Meloen RH, van der Eb AJ, Melief CJM: Eradication of adenovirus E1-induced tumours by E1A-specific cytotoxic T lymphocytes. Cell 59: 603-614, 1989.

17. Engelhard VH: Structure of peptides associated with class I and class II MHC molecules. Annu Rev Immunol 12:181-207, 1994.

18. De Bruyn MLH, Schumacher TNM, Nieland JD, Ploegh HL, Kast WM, Melief CJM: Peptide loading of empty major histocompatibility complex molecules on RMA-S cells allows the induction of

primary cytotoxic T lymphocyte responses. Eur J Immunol 21: 2963-2970, 1991.

19. van der Burg SH, Ras E, Drijfhout JW, Benckhuijsen WE, Bremers AJA, Melief CJM, Kast WM: An HLA class I peptide-binding assay based on competition for binding to class I molecules on intact human B-cells: identification of conserved HIV-1 polymerase peptides binding to HLA*0301. Human Immunology, *in press*.

20. van der Burg SH, Visseren MJW, Brandt RMP, Kast WM, Melief CJM: Immunogenicity of peptides bound to MHC class I molecules depends on the MHC-peptide complex stability. *Submitted*.

21. Nijman HW, Houbiers JGA, Vierboom MPM, van der Burg SH, Drijfhout JW, D'Amaro J, Kenemans P, Melief CJM, Kast WM: Identification of peptide sequences that potentially trigger HLA-A2.1-restricted cytotoxic T lymphocytes. Eur J Immunol 23: 1215-1219, 1993.

22. Taylor WR: Identification of protein sequence homology by consensus template alignment. J Mol Biol 188: 233-258, 1986.

23. Gausepohl H, Frank RW: Automatische multiple peptide-synthese. BioTec. (September 1990).

24. Gausepohl H, Kraft M, Boulin C, Frank RW: In: E Giralt and D Andreu (eds). Peptides: 206-207, 1990.

25. Rapp W, Zhang L, Bayer E: Continuous flow peptide synthesis on PSPOE-Graft-copolymers. In: Innovation and perspectives in solid phase peptide synthesis: 205-210, 1990.

26. Sheppard RC, Williams BJ: Acid-labile resin linkage agents for use in solid phase peptide synthesis. Peptide Protein Res 20: 451-454, 1982.

27. Gausepohl H, Kraft M, Frank R: In situ activation of FMOC-amino acids by BOP in solid phase peptide synthesis. Peptides: 241-243, 1988.

28. Castro B, Dormoy JR, Evin G, Selve C: Reactifs de couplage peptidique IV (1)-L'hexafluorophosphate de benzotriazolyl N-oxytrisdimethylamino phosphonium (B.O.P.). Tetrahedron Letters 14: 1219-1222, 1975.

29. Fields GB, Noble RL: Solid phase peptide synthesis utilizing 9-fluorenylmethoxycarbonyl amino acids. Int J Peptide Protein Res 35: 161-214, 1990.

SEQUENCE LISTING

SEQ ID NO:1
SEQUENCE TYPE: amino acid
SEQUENCE LENGTH:9
GlnValLeuAlaPheGlyLeuLeuLeu
1 5 9

SEQ ID NO:2
SEQUENCE TYPE: amino acid
SEQUENCE LENGTH:11
TyrGlnLeuAspProLysPheIleThrSerIle
1 5 10 11

SEQ ID NO:3
SEQUENCE TYPE: amino acid
SEQUENCE LENGTH:9
IleLeuTyrGluAsnAsnValIleThr
1 5 9

SEQ ID NO:4
SEQUENCE TYPE: amino acid
SEQUENCE LENGTH:9
GlyLeuLysAlaGlyValIleAlaVal
1 5 9

SEQ ID NO:5
SEQUENCE TYPE: amino acid
SEQUENCE LENGTH:9
ValValAlaGlyIleValValLeuVal
1 5 9

CLAIMS

1. A peptide comprising an amino acid sequence which has the ability to bind to a human Major Histocompatibility Complex (MHC) Class I molecule, wherein said amino acid sequence is derived from the human epithelial antigen Ep-CAM.

2. A peptide according to claim 1, wherein said Ep-CAM-derived amino acid sequence has the ability to bind to the human MHC Class I allele HLA-A*0201.

3. A peptide according to claim 1 or claim 2, which peptide is capable of inducing a primary cytotoxic T lymphocyte (CTL) response.

4. A peptide according to any one of the aforegoing claims, said peptide comprising one of the following sequences:

Q V L A F G L L L (SEQ ID NO:1)
Y Q L D P K F I T S I (SEQ ID NO:2)
I L Y E N N V I T (SEQ ID NO:3)
G L K A G V I A V (SEQ ID NO:4)
V V A G I V V L V (SEQ ID NO:5)

or a fragment, homolog, isoform, derivative, genetic variant or conservative variant of any one of these amino acid sequences which has the ability to bind to the human MHC Class I allele HLA-A*0201.

5. A peptide according to claim 4, wherein said sequences are flanked on either side or on both sides by amino acid residues constituting a processing site for a cell which carries the MHC Class I HLA-A*0201 allele.

6. A peptide according to any one of the aforegoing claims, said peptide comprising 8-12 amino acids.

7. A peptide which consists of one of the following sequences:

Q V L A F G L L L (SEQ ID NO:1)
Y Q L D P K F I T S I (SEQ ID NO:2)
I L Y E N N V I T (SEQ ID NO:3)
G L K A G V I A V (SEQ ID NO:4)
V V A G I V V L V (SEQ ID NO:5)

or one of these sequences in which one or more of the residues are replaced by an alternative residue not altering the binding to the MHC Class I HLA-A*0201 molecule.

8. A peptide according to any one of the aforegoing claims for use as a medicine or as a diagnostic reagent.

9. A peptide according to any one of the aforegoing claims for use in the treatment or prophylaxis of Ep-CAM positive tumor cells or other diseases where Ep-CAM positive cells are affected.

10. Use of a peptide according to any one of claims 1-8 in the preparation of a pharmaceutical composition for the treatment or prophylaxis of Ep-CAM positive tumor cells or other Ep-CAM related disease.

11. A pharmaceutical composition comprising a peptide according to any one of claims 1-8 and a pharmaceutically acceptable carrier or diluent.

12. A vaccine preparation for prophylaxis or treatment of carcinoma comprising a peptide according to any one of claims 1-8 and a suitable adjuvant.

13. Use of a peptide according to any one of claims 1-8 in inducing a CTL response.

14. Use according to claim 13, wherein the response is a HLA class I restricted CD8-positive cytotoxic T cell response.

15. A method of prophylactic or therapeutic treatment of Ep-CAM positive conditions in a human, comprising administering to said human individual a prophylactically or therapeutically effective amount of an immunogenic form of a peptide according to any one of the claims 1-8.

INTERNATIONAL SEARCH REPORT

Inte Application No
PCT/NL 96/00414

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C07K14/705 A61K38/17 A61K39/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP 0 326 423 A (ELI LILLY) 2 August 1989 * pages 2, 8, 16 * ---	1-5
X	MAT.RES.SOC.SYMP.PROC., vol. 292, page 205-210 XP000567100 DEGUCHI, Y. ET AL.: "Synthesis and characterization of periodic polypeptides ..." * page 4, compound 3 * ---	5
Y	PROC.NATL.ACAD.SCI. USA, vol. 87, pages 3542-3546, XP000566331 SZALA, S. ET AL.: "Molecular cloning of cDNA for the carcinoma-associated antigen GA733-2" * figure 3 * --- -/--	1-15

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

1

Date of the actual completion of the international search	Date of mailing of the international search report
19 February 1997	05.03.97

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax (+ 31-70) 340-3016	Hermann, R

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Int. Application No
PCT/NL 96/00414

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	ANNU.REV.IMMUNOL., vol. 12, pages 181-207, XP000566629 ENGELHARD, V.H.: "Structure of peptides associated with class I and class II MHC molecules" * abstract; p. 198 *	1-15
A	EP 0 252 741 A (CENTOCOR, INC.) 13 January 1988	

1

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Information on patent family members

Intl. Application No
PCT/NL 96/00414

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
EP-A-326423	02-08-89	DE-D-	68922757	29-06-95
		DE-T-	68922757	16-11-95
		JP-A-	2005867	10-01-90
		US-A-	5348887	20-09-94
EP-A-252741	13-01-88	EP-A-	0755683	29-01-97
		JP-A-	63060941	17-03-88